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Protein kinase C regulates adenosine A2a receptor mRNA expression in SH-SY5Y cells

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Abstract

Protein kinase C regulates mRNAs encoding several G protein-linked receptors but its role in adenosine A2a receptor expression is not known. We tested the hypothesis that protein kinase C activated by tetradecanoyl phorbol acetate (TPA) regulates adenosine A2a receptor mRNA levels. SH-SY5Y human neuroblastoma cells express adenosine receptors which positively couple to adenylyl cyclase with a pharmacologic profile expected of the A2a subtype. Northern blotting demonstrated an adenosine A2a receptor mRNA species of similar molecular size in SH-SY5Y cells and in human brain. TPA increased adenosine A2a receptor mRNA in a dose- and time-dependent fashion. Transcription or translation inhibition prevented increases in adenosine A2a receptor mRNA. Bisindolylmaleimide blocked TPA effects. Adenosine A2a receptor mRNA stability was unchanged by TPA. This study identifies a human neuroblastoma cell line expressing functional adenosine A2a receptors. Protein kinase C activation appears to enhance transcription of the adenosine A2a receptor gene. © 1997 Elsevier Science B.V.

Keywords: Adenosine receptor; Phorbol ester; Protein kinase C; SH-SY5Y neuroblastoma cell; cAMP

1. Introduction

Adenosine receptors of the A2a subtype are members of the G protein coupled receptor superfamily. Adenosine A2a receptors couple to G_s and stimulate the formation of cAMP in target cells (Fredholm et al., 1994). Regulation of the adenosine A2a receptor has been studied at the level of the receptor protein in vivo (Hawkins et al., 1988; Porter et al., 1988), and in cell lines which express the endogenous (Ramkumar et al., 1991; Chern et al., 1993) and transfected (Palmer et al., 1994) adenosine A2a receptor. These studies indicate that agonist-induced desensitization of the adenosine A2a receptor is mediated by multiple processes, including phosphorylation and internalization of the receptor protein along with alterations in the activities of enzymes in the receptor's signal transduction pathway (Olah and Stiles, 1995). Similar mechanisms have been shown to regulate responsiveness of β-adrenoceptors (Hausdorff et al., 1990).

Regulation of adenosine A2a receptor mRNA has been described in PC12 cells (Saitoh et al., 1994). The adenosine receptor agonist 5′-*N*-ethylcarboxyamidoadenosine (NECA) produced an initial increase in adenosine A2a receptor mRNA levels followed by a sustained decline, and then a gradual recovery. Activation of the cAMP signal transduction pathway through adenylate cyclase and protein kinase A was implicated in the mechanism of adenosine A2a receptor mRNA regulation by adenosine A2a receptor agonists. This regulation of adenosine A2a receptor mRNA levels resembles that of β-adrenoceptor mRNA levels (Hadcock et al., 1989; Hosoda et al., 1995) and cAMP mediated regulation of mRNA for other G protein coupled receptor subtypes (Fujimoto and Gershengorn, 1991; Wang et al., 1991b).

In addition to protein kinase A mediated signal transduction, the role of the signal transduction pathway mediated by the protein kinase C family of serine–threonine kinases has been studied in the regulation of adenosine receptors. cAMP generation in response to adenosine receptor agonists is attenuated when phorbol esters are used to activate protein kinase C in PC12 cells (Horwitz, 1989).

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Although mRNAs for several other G protein-coupled receptor subtypes are regulated by protein kinase C (Fujimoto et al., 1991; Ferry et al., 1994; Feve et al., 1995), it is not known whether activation of protein kinase C affects adenosine receptor mRNAs. Complex regulatory influences, including those activating protein kinase C-dependent pathways, act on brain, cardiovascular and immune cells which express adenosine A2a receptors. In this study we tested the hypothesis that adenosine A2a receptor expression is regulated by protein kinase C. We used selective adenosine A2a receptor ligands, i.e. 2-[4-(2-carboxyethyl)phenylethylamino]-5′-*N*-ethylcarboxyamidoadenosine (CGS 21680) (Jarvis et al., 1989) and 2-chlorostyrylcaffeine (CSC) (Jacobson et al., 1993), and selective molecular probes resulting from our characterization of the human adenosine A2a receptor gene (Peterfreund et al., 1996), to show that SH-SY5Y human neuroblastoma cells express adenosine A2a receptor mRNA and functional adenosine A2a receptors. We demonstrate that adenosine A2a receptor mRNA levels in SH-SY5Y cells increase in response to phorbol ester treatment in a dose-, time- and structure-dependent fashion. The effects of phorbol ester treatment are prevented by a selective protein kinase C inhibitor, and by cycloheximide and actinomycin D, and do not depend on changes in mRNA stability.

2. Materials and methods

2.1. Reagents

Adenosine receptor agonists NECA, CGS 21680, 5′-(*N*-cyclopropyl)-carboxamidoadenosine (CPCA) and N^6 cyclohexyladenosine (CHA), antagonists 3,7-dimethyl-1-propargylxanthine (DMPX) and 8-(3-chlorostyryl) caffeine (CSC), and the phosphodiesterase inhibitor 4-[(3-butoxy-4-methoxyphenyl)methyl]-2-imidazolidinone (Ro20-1724) were obtained from Research Biochemicals International (Natick, MA, USA). Bisindolylmaleimide was from Calbiochem (La Jolla, CA, USA). Phorbol esters, forskolin, actinomycin D and cycloheximide were from Sigma (St. Louis, MO, USA). Restriction endonucleases and RNA polymerases were obtained from Gibco-BRL (Grand Island, NY, USA) or Promega (Madison, WI, USA). An oligonucleotide labeling kit using the Klenow fragment of DNA polymerase was obtained from Pharmacia (Uppsala, Sweden). Radionucleotides were obtained from Dupont-NEN (Boston, MA, USA). Cell culture media and reagents were from Gibco. Fetal bovine serum was from Sigma. Other reagents were of molecular biology grade.

2.2. Cell culture

SH-SY5Y human neuroblastoma cells were maintained in monolayer culture in Dulbecco's Modified Eagle Medium supplemented with fetal bovine serum (10%, v/v) at 37°C in an atmosphere of 5% CO_2 on standard tissue culture plastic dishes. Cells were passaged at weekly intervals by gentle trypsinization. For cAMP generation experiments, cells were seeded into 6-well plates at a density of 1×10^6 cells per 35 mm well and studied when confluent, 48–72 h later. For RNA experiments, cells were seeded at 1×10^6 cells per 100 mm plate, and studied after one week by which time the monolayer was just subconfluent. We observed that cAMP generation in response to adenosine A2a receptor agonists declined with increasing passage number. Consequently, experiments were carried out with cells at passage number 15 or lower.

2.3. cAMP generation studies

The serum-containing cell culture medium was replaced with serum-free medium 4–16 h before drug treatment. Fresh serum-free medium was added 1–2 h before experiments. Control dishes were treated with vehicle alone (water, dimethyl sulfoxide (DMSO) or ethanol). Adenosine receptor antagonists were usually added 10 min before agonists. In a pilot experiment we observed that addition of a phosphodiesterase inhibitor was necessary to preserve cAMP generated in response to agonists. Therefore the phosphodiesterase inhibitor Ro20-1724 (50 μM final concentration) was added immediately before addition of control solutions or agonists. Agonist incubations lasted for 10 min after which the medium was aspirated and the dishes washed once with ice cold phosphate buffered saline. Cells were then scraped from the wells in an ice cold solution of acetic acid (50 mM) and EDTA (5 mM). The cell extract was boiled for 2–3 min, chilled on ice and centrifuged at $14\,000 \times g$ for 2 min in the cold room. The supernatant was collected, dried and stored frozen for cAMP assay using a competitive protein binding kit (Amersham Life Sciences, Buckinghamshire). The pellet was saved for protein assay (BCA Protein Assay Reagent Kit, Pierce, Rockford, IL, USA). Experiments were performed in duplicate or triplicate. Data is reported from at least three separate experiments.

2.4. RNA studies

Sections of frozen post mortem human brain (provided by Dr. Edward Bird, McLean Hospital Brain Tissue Resource Center, Belmont, MA, USA) were dissected according to a Brodmann map. After homogenization, total RNA was extracted with guanidinium isothiocyanate and purified over a gradient of cesium chloride ($CsCl_2$). The integrity of the RNA was confirmed by examining an aliquot in a nondenaturing ethidium agarose gel. The RNA concentrations were determined by UV absorbance at 260 nm. SH-SY5Y cells received fresh medium 1 h before the administration of experimental treatments or vehicle controls. At the end of the incubation period, the medium was aspirated and the cells washed once with ice cold phos-

phate buffered saline. The cells were then scraped from the plate in a small volume of the same solution, pelleted ($14\,000 \times g$ for 2 min in the cold room) and stored at −80°C. Total RNA was prepared from the pellet by centrifuging a guanidine isothiocyanate extract of the cells over a $CsCl_2$ gradient. The RNA (10 μg per lane) was size fractionated on a 1.1% agarose gel, transferred to GeneScreen nylon membranes (Dupont-NEN) by electroblotting, fixed by UV light and prehybridized in a solution of 50% (v/v) formamide, 1% (v/v) sodium dodecyl sulfate, 1 M NaCl with 10% (w/v) dextran and 10 × Denhardt's solution buffered with Tris–HCl and supplemented with 100–200 μg/ml boiled, sheared salmon sperm DNA at 65°C (adenosine A2a receptor cRNA probe) or 42°C (actin cDNA probe). Membranes were hybridized in the same solution for 16–24 h. Membranes hybridized with the cRNA probe were briefly rinsed in 2 × SSC (SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0) at room temperature followed by sequential washing in 2 × SSC/0.1% sodium dodecylsulfate (SDS) at 75°C, and 0.1 × SSC/0.1% SDS at 75°C. Blots hybridized with the actin cDNA probe were rinsed in 2 × SSC at room temperature followed by sequential washing in 2 × SSC/1% SDS at 65°C and then in 0.2 × SSC at room temperature. All washes were for 60 min with one change of solution. Membranes were exposed to Kodak XAR film (Eastman Kodak, Rochester, NY, USA) at −80°C with one intensifying screen for 2–8 h (actin probe) and 16–72 h (adenosine A2a receptor probe). Quantitative autoradiographic data were obtained by densitometry of the scanned images with the program NIH Image. Data reported represent the results of at least two independent experiments performed in triplicate or quadruplicate.

2.5. Preparation of hybridization probes

The human adenosine A2a receptor fragment pHUAD2 cloned into plasmid Bluescript II (Stratagene, La Jolla, CA, USA) was the template for cRNA synthesis. This fragment was derived from a human adenosine A2a receptor-containing cosmid and was characterized by sequencing (MacCollin et al., 1994; Peterfreund et al., 1996). pHUAD2 spans 735 bp from a *Pst*I restriction endonuclease digestion site located 5′ to the sequence encoding putative transmembrane domain VII to a second *Pst*I site in the 3′ untranslated region of the reported human adenosine A2a receptor cDNA (Furlong et al., 1992). Fragment pHUAD2 encodes the entire human adenosine A2a recep-

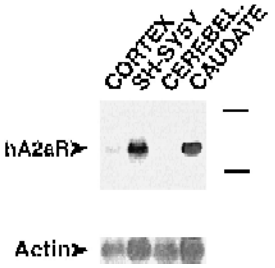

Fig. 1. Northern blot analysis of SH-SY5Y RNA and human brain tissues. The selective human adenosine A2a receptor hybridization probe was pHUAD2. The label hA2aR indicates the signal from the human adenosine A2a receptor probe in this and all other figures. RNA loading was 15 μg/lane. Exposure of the lanes containing human caudate and cerebellum RNA was for 1 h. Exposure of the remaining lanes was for 6 h. Only faint autoradiographic signals were seen for cerebellum in lengthy exposures (not shown). The blot was rehybridized to a probe for human β-actin. The data is representative of three similar experiments.

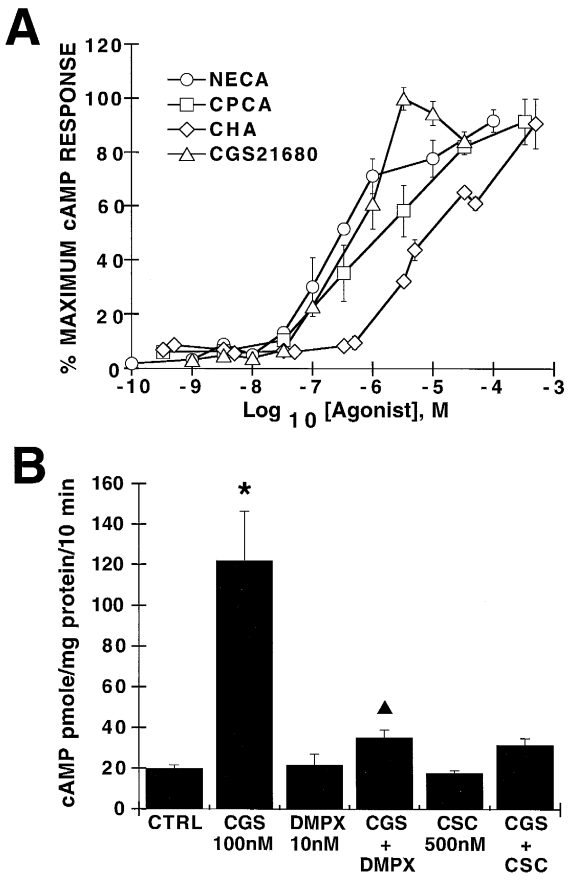

Fig. 2. Response of SH-SY5Y cells to adenosine receptor agonists and antagonists. (A) Production of cAMP in response to increasing doses of adenosine receptor agonists. Curves represent mean response ± S.E.M. of pooled data from 3–5 individual experiments, normalized to the maximum response in each experiment. The absolute value of cAMP produced by a maximal dose of NECA or CGS 21680 ranged from 200–500 pmol cAMP/mg protein/10 min with control values 10–25 pmol cAMP/mg protein/10 min. (B) CGS 21680 stimulated cAMP production, effect of adenosine receptor antagonists. A submaximal dose of CGS 21680 was used to stimulate cAMP production in the presence of 3,7-dimethyl-1-propargylxanthine (DMPX) or 8-(3-chlorostyryl) caffeine (CSC). Bars represent mean response ± S.E.M. of pooled data from at least three separate experiments, $n = 6$–12 per point. Treatment data were compared using ANOVA with a Tukey-Kramer Multiple Comparisons Test to allow comparisons between individual groups. ▲ Indicates different from control, $P < 0.05$. * Indicates different from control, $P < 0.01$. The differences in response between the CGS 21680 alone group and the CGS 21680 + antagonist groups were significant, $P < 0.001$. DMPX (1 nM) and CSC (50 nM) both antagonized the CGS 21680 response approx. 50% (not shown).

tor carboxy terminus, which is much longer than those of the other known adenosine receptors (Linden et al., 1993), and has a low nucleotide sequence homology to other receptors. The plasmid containing pHUAD2 was linearized in the polylinker and purified by phenol extraction and ethanol precipitation for use as a cRNA template. cRNA synthesis utilized T3 RNA polymerase and [$^{[32]}$P]CTP. The $^{[32]}$P-labeled cRNA was purified over a Nuc Trap gel filtration column (Stratagene). 2×10^6 cpm of the cRNA probe were added per ml of hybridization solution. The human actin probe fragment was obtained from Clontech (Palo Alto, CA, USA) and labeled by the random priming method with the large fragment of DNA polymerase I (Klenow fragment) to a specific activity of $0.5–1 \times 10^9$ cpm/μg. Labeled fragments were purified over a gel filtration column (NICK column, Pharmacia Biotech). The hybridization solution contained 100 000–200 000 cpm/ml of the labeled actin probe.

2.6. Data and statistical analysis

For cAMP and protein assays, standard curves were prepared with known concentrations of reagents and the data analyzed by linear regression using the program Instat 2.0 (GraphPad, Sorrento Valley, CA, USA). Raw experimental data were then analyzed with the standard curve. cAMP values were normalized for the recovery of protein in the samples and the results analyzed by analysis of variance (ANOVA). For quantitative autoradiographic data, the specific adenosine A2a receptor densitometry value was normalized to the value for β-actin. The results were pooled and analyzed as described in the figure legends.

3. Results

3.1. Expression of adenosine A2a receptor mRNA in SH-SY5Y cells

In Northern blots using the adenosine A2a receptor probe pHUAD2, SH-SY5Y cells express a single RNA species which co-migrates with the hybridization signal from control human brain tissue (Fig. 1). The level of adenosine A2a receptor mRNA expression in SH-SY5Y cells is lower than that found in samples of human basal ganglia, the tissue with the most abundant adenosine A2a receptor mRNA expression (Peterfreund et al., 1996). However, adenosine A2a receptor mRNA levels are read-

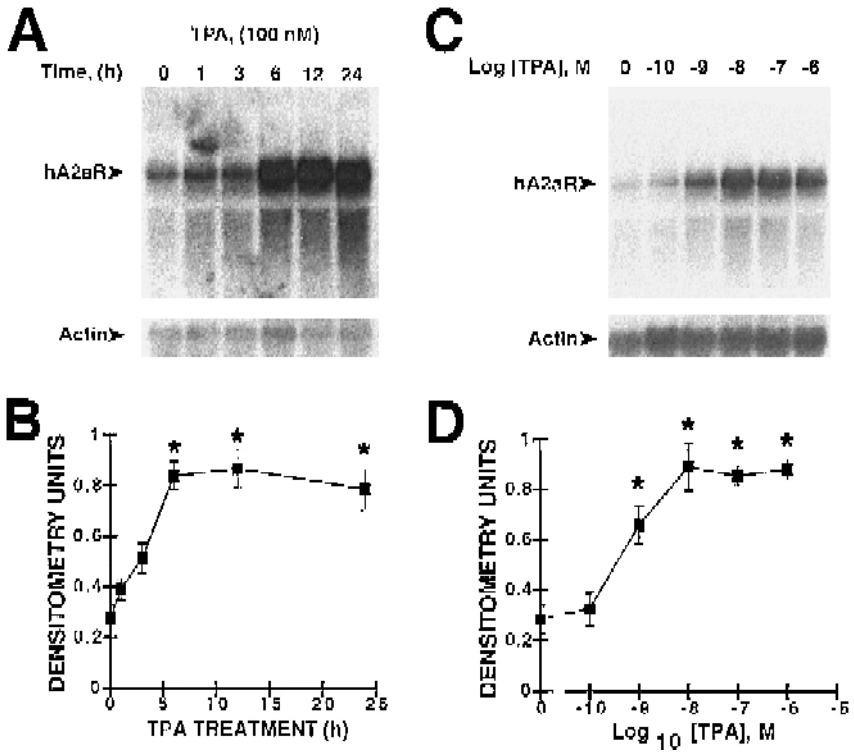

Fig. 3. Adenosine A2a receptor mRNA levels following TPA treatment. (A) Northern blot analysis. TPA (100 nM) was administered for 1–24 h. Data is representative of three independent experiments performed in duplicate or triplicate. (B) Quantitative analysis of the Northern blot TPA time response data. Densitometry data was normalized to the maximum response in each experiment, pooled, and plotted as the mean ± S.E.M. of 6–7 determinations. The results were evaluated by the Dunnett Multiple Comparison Test using the vehicle treatment group as the control. * Indicates different from control, $P < 0.01$. Other treatments were not significantly different from control, $P > 0.05$. (C) Northern blot analysis of increases in adenosine A2a receptor mRNA levels in response to increasing doses of TPA. Incubation time was 6 h. Data is representative of replicate experiments performed in triplicate or quadruplicate. (D) Quantitative analysis of the Northern blot TPA dose response data. Densitometry data were evaluated as described for panel B.

ily detectable under basal conditions in the SH-SY5Y cell line thereby providing the opportunity for analysis of adenosine A2a receptor gene regulation.

3.2. Characterization of functional adenosine A2a receptors

To confirm that expression of adenosine A2a receptor mRNA by the SH-SY5Y cells correlates with the presence of functional receptors, we measured production of the second messenger cAMP in response to selective adenosine receptor agonists (Fig. 2A). cAMP was detected in extracts of SH-SY5Y cells before the addition of adenosine receptor agonists. Adenosine receptor agonists increased production of cAMP in a dose-dependent fashion. The maximum increase in cAMP by adenosine receptor agonists was approx. 20-fold over basal levels. The rank order of potency of agonists was NECA > CGS 21680 > CPCA > CHA, typical of the profile expected for an adenosine A2a receptor (Fredholm et al., 1994). Administration of the adenosine receptor antagonists 3,7-dimethyl-1-propargylxanthine (DMPX) or 8-(3-chlorostyryl) caffeine (CSC) blocked CGS 21680-dependent increases in cAMP (Fig. 2B), confirming that the cAMP responses to adenosine agonist stimulation are receptor mediated.

3.3. Regulation of adenosine A2a receptor mRNA levels by TPA administration

To test the hypothesis that the level of adenosine A2a receptor mRNA, like that of other G protein-coupled receptors, is subject to regulation by phorbol esters, adenosine A2a receptor mRNA levels were measured after treatment with tetradecanoyl phorbol acetate (TPA,100 nM) (Fig. 3A and B). TPA treatment produced a time-dependent elevation of adenosine A2a receptor mRNA. Adenosine A2a receptor mRNA elevation was maximal by 6 h of incubation and sustained for 24 h, the longest timepoint studied. The maximum increase in adenosine A2a receptor mRNA induced by TPA was 2–4-fold. The response to TPA was dose-dependent with a maximum response at 10 nM (Fig. 3C and D). Incubations of SH-SY5Y cells with ethanol (24 h), the solvent for TPA, or dimethyl sulfoxide, an alternate solvent, did not alter adenosine A2a receptor mRNA levels (data not shown).

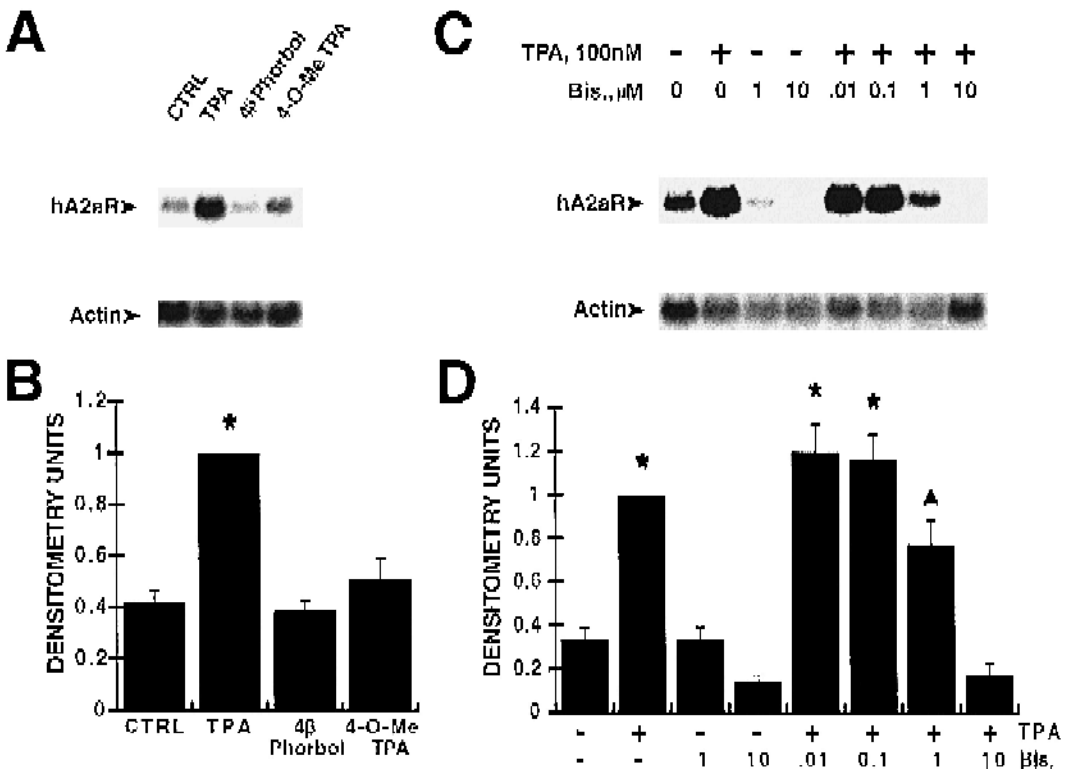

Fig. 4. Specificity of the response to phorbol esters. (A) Northern blot analysis of the response of adenosine A2a receptor mRNA to TPA and two phorbol analogs. Phorbol compounds (100 nM) were administered for a 6 h incubation period. Data is representative of three independent experiments performed at least in triplicate. (B) Quantitative analysis of the Northern blot phorbol analog response data. Densitometry data was analyzed by ANOVA with a Dunnet's test for comparisons between treatment groups. The data represent the pooled mean ± S.E.M. after normalizing the results in each experiment to the response to TPA. * Indicates different from control, $P < 0.01$. Other treatments were not significantly different from control, $P > 0.05$. (C) The selective protein kinase C antagonist bisindolylmaleimide inhibits TPA effects. Cells were pretreated with vehicle (DMSO) or bisindolylmaleimide (0.01 μM to 10 μM) for 20–30 min before administration of TPA or ethanol control for 6 h. Data is representative of replicate experiments performed in triplicate. (D) Quantitative analysis of the Northern blot TPA + bisindolylmaleimide dose response data. Densitometry data were evaluated and plotted as described for panel B. * Indicates different from control, $P < 0.01$. ▲ Indicates different from control, $P < 0.05$. Other treatments were not significantly different from control, $P > 0.05$.

3.4. Specificity of the regulatory effects of TPA on adenosine A2a receptor mRNA

To investigate whether the actions of TPA on adenosine A2a receptor mRNA levels result from nonspecific effects of phorbol derivatives, SH-SY5Y responses to phorbol compounds known to be inactive in other systems were assessed. The inactive phorbol compounds 4β phorbol and 4-O–Me TPA (both at 100 nM) failed to increase adenosine A2a receptor mRNA levels (Fig. 4A and B). The efficacy of TPA and the lack of response to the other phorbol compounds is consistent with established structure activity profiles (Hecker, 1978). Because the protein kinase C family of serine–threonine kinases is believed to mediate biological actions of phorbol esters and related compounds, we tested the effects of a selective inhibitor of protein kinase C activity, bisindolylmaleimide, on the response to TPA. Bisindolylmaleimide produced a dose-dependent inhibition of the responses to TPA (Fig. 4C and D).

3.5. Mechanisms regulating adenosine A2a receptor mRNA levels

To determine whether the effect of TPA on adenosine A2a receptor mRNA is due to increased stability of adenosine A2a receptor mRNA, we measured adenosine A2a receptor mRNA in the presence or absence of TPA following transcription inhibition with actinomycin D (Fig. 5A and B). The decay curves for adenosine A2a receptor mRNA levels were superimposable for SH-SY5Y cells receiving TPA or the ethanol vehicle control followed by the administration of actinomycin. This result indicates that TPA does not alter the stability of adenosine A2a receptor mRNA. The apparent adenosine A2a receptor mRNA half life was 4–5 h. Pretreatment with actinomycin D inhibited the TPA induced increase in adenosine A2a receptor mRNA, consistent with a role for transcription in the actions of TPA on adenosine A2a receptor gene expression in SH-SY5Y cells (Fig. 6A and B).

The role of protein synthesis in TPA responses was investigated by treating SH-SY5Y cells with cycloheximide. Administration of cycloheximide alone was associated with increases in adenosine A2a receptor mRNA (Fig. 6C and D). Similar effects were observed with another protein synthesis inhibitor, anisomycin (not shown). Combining TPA with cycloheximide failed to produce levels of adenosine A2a receptor mRNA greater than those obtained with cycloheximide alone. These data suggest a role for protein synthesis in the actions of TPA on adenosine A2a receptor mRNA. Co-administration of actinomycin D and either cycloheximide or anisomycin resulted in no increases in adenosine A2a receptor mRNA levels (control, 0.26 ± 0.018; anisomycin, 0.82 ± 0.039; anisomycin/actinomycin, 0.14 ± 0.036; cycloheximide, 1.27 ± 0.11; cycloheximide/anisomycin, 0.14 ± 0.02; arbitrary densitometry units, $n = 3$/group; only the anisomycin alone and cycloheximide alone treatment groups were significantly different from control, $P < 0.01$). The data suggest the existence of a labile repressor protein which acts to reduce adenosine A2a receptor mRNA levels.

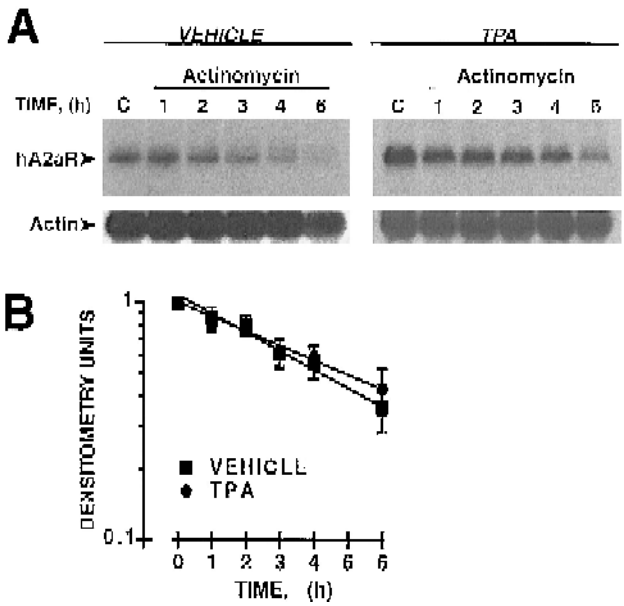

Fig. 5. Determination of adenosine A2a receptor (hA2aR) mRNA decay half-life. (A) Northern blot analysis. Dishes of SH-SY5Y cells were treated for 3 h with TPA or ethanol vehicle control, after which actinomycin D (5 μg/ml) was added ($t = 0$). Samples were harvested at the indicated time points up to 6 h from the time of the addition of the transcription inhibitor. (B) Quantitative determination of decay half-life. Data were pooled from 6 independent experiments and analyzed by linear regression. The slopes of the decay curves were not significantly different (TPA, -0.1343 ± 0.0107 (95% confidence interval, -0.1639 to -0.1047); Vehicle, -0.1670 ± 0.0110 (95% confidence interval, -0.1976 to -0.1363)). Therefore, the estimated decay half-life for the TPA treated cells (5.16 h) was not different from that of the vehicle treated cells (4.15 h).

4. Discussion

Several reports describe the expression of adenosine receptors positively coupled to adenylate cyclase in human cells. Stimulation of IMR32 human neuroblastoma cells with adenosine receptor agonists increased formation of cAMP (Abbracchio et al., 1989). However, the pharmacologic data provided in that study do not permit a clear distinction between expression of the A2a and A2b subtypes of adenosine A2 receptors. In human D384 astrocytoma cells, the rank order of potency for adenosine receptor agonists to stimulate cAMP formation was consistent with the presence of A2b receptors, rather than adenosine A2a receptors (Altiok et al., 1992). Similarly, the human lung fibroblast line VA13 cells expresses an A2b-like receptor (Rivkees and Reppert, 1992). With subtype selective adenosine receptor agonists and antagonists, co-ex-

pression of both adenosine A2a and A1 receptors was demonstrated in human fibroblast cells (Ahmed et al., 1995). In the present study, we show that the SH-SY5Y human neuroblastoma cell line expresses an mRNA species of appropriate molecular size which hybridizes to a specific adenosine A2a receptor probe under stringent conditions. Furthermore, the profile of cAMP generation in response to adenosine receptor agonists and antagonists is consistent with the functional expression of adenosine receptors of the A2a subtype. To our knowledge, the present study is the first to suggest the presence of adenosine A2a receptors coupled to adenylate cyclase in a human neural cell line.

We tested the hypothesis that levels of adenosine A2a receptor mRNA were subject to regulation by TPA in SH-SY5Y cells. Phorbol ester treatment was associated with a dose-, structure- and time-dependent increase in adenosine A2a receptor mRNA levels. The effective doses of TPA were within the range of active doses observed in other experimental systems, 10 nM–1 μM. The structure–activity profile for phorbol ester analogs obtained in this study is also consistent with established concepts of phorbol ester pharmacology (Hecker, 1978). The increase in adenosine A2a receptor mRNA found in SH-SY5Y cells was not observed until after several hours of treatment, but was sustained. This time–response profile differs from the rapid, transient increases of 5HT2A mRNA induced by phorbol esters in P11 cells (Ferry et al., 1994). Furthermore, phorbol ester treatment of SH-SY5Y cells elevates levels of adenosine A2a receptor mRNA, in contrast to the decreases in mRNA levels for the TRH receptor observed in the GH3 cell line (Fujimoto et al., 1991), the β3-adrenoceptor seen in 3T3 cells (Feve et al., 1995) and the LH/CG receptor reported in a Leydig cell line (Wang et al., 1991a). Thus the phenomenon of phorbol ester induced changes in G protein-coupled receptor mRNA levels is not a general pharmacologic effect. Instead, phorbol ester regulation depends on the particular receptor gene and cell.

The traditionally accepted cellular target for biologi-

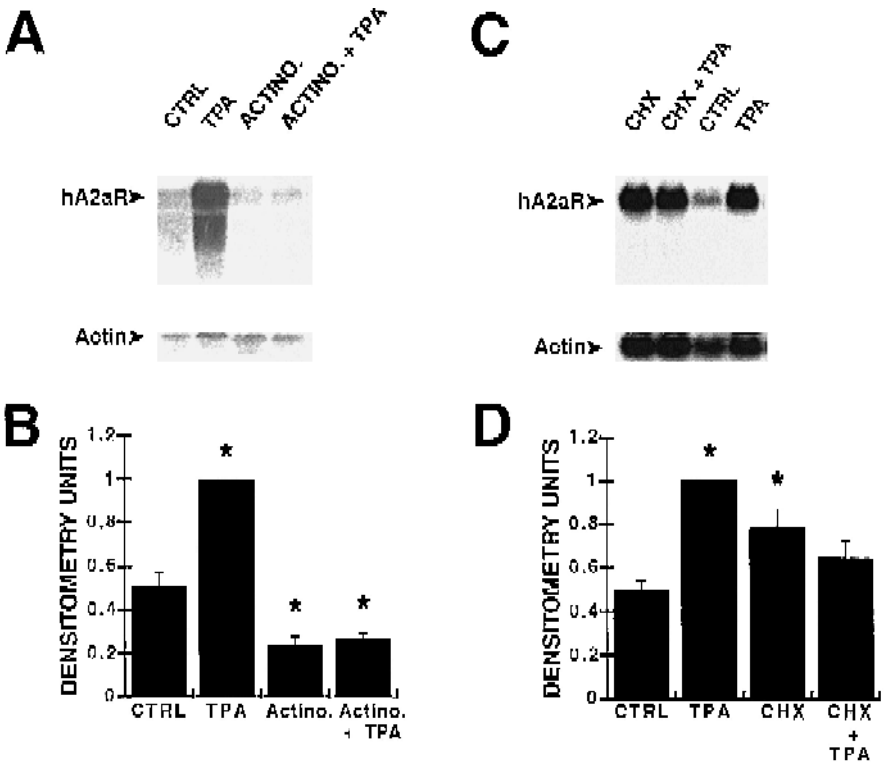

Fig. 6. Transcription and translation dependence of adenosine A2a receptor (hA2aR) mRNA responses to TPA. (A) Northern blot analysis: Effect of inhibition of transcription. Cells received actinomycin D (5 μg/ml) 20–30 min before TPA (100 nM) or vehicle then incubated for 6 h. To emphasize bands with low signal intensity, the blot was exposed for 48 h. The data is representative of three similar experiments performed in triplicate. (B) Quantitative analysis of the Northern blot transcription inhibition data. Densitometry data was analyzed by ANOVA with a Tukey–Kramer test for comparisons between treatment groups. The data represent the pooled mean ± S.E.M after normalizing the results in each experiment to the response to TPA. * Indicates different from control, $P < 0.01$. Actinomycin D suppressed the response to TPA alone, $P < 0.01$. There was no significant difference ($P > 0.05$) between the actinomycin D alone and the actinomycin D plus TPA groups. (C) Northern blot analysis: Effect of inhibition of translation. Cells received cycloheximide (CHX) (100 μM) 20–30 min before TPA or vehicle then incubated for 6 h. Similar effects were obtained with anisomycin D (not shown). Representative of three similar experiments performed in triplicate. (D) Quantitative analysis of the Northern blot translation inhibition data. Data were evaluated as described for (B). * Indicates different from control, $P < 0.01$. Cycloheximide suppressed the response to TPA alone, $P < 0.01$. There was no significant difference ($P > 0.05$) between the cycloheximide alone and the cycloheximide plus TPA groups.

cally active phorbol esters is the family of enzymes known as protein kinase C. Members of the protein kinase C family share common structural features including a cysteine rich amino terminus (C1), an ATP binding domain (C3) and the carboxy terminal catalytic region (C4). The α, βI, βII and γ isoforms contain an additional domain for calcium binding (C2). This region is absent in other family members (Burns et al., 1992). Binding of diacylglycerol or biologically active phorbol esters to the C1 region activates the enzyme, apparently via a conformational change. Calphostin C blocks binding of lipid activators to this regulatory region of protein kinase C family members (Kiley and Jaken, 1994). Inhibitors of the ATP binding domain include H7 and staurosporine, however these agents are nonselective for inhibiting protein kinase C and other kinases whereas the staurosporine homolog bisindolylmaleimide is highly selective for protein kinase C relative to other kinases (Toullec et al., 1991). Recently, the cysteine rich proteins Unc 13 from C. elegans and the mammalian GTPase *n*-chimaerin have been shown to bind phorbol compounds in a calphostin C sensitive manner (Kiley and Jaken, 1994). Consequently, interpretation of studies in which the actions of phorbol esters are prevented by H7, staurosporine or calphostin C is potentially confounded by actions on targets other than protein kinase C.

SH-SY5Y human neuroblastoma cells exhibit several cellular responses to the administration of phorbol esters. One such response is enhancement of calcium channel currents independent of protein kinase C activation (Reeve et al., 1995). There is also phenotypic maturation independent of several isoforms of protein kinase C (Jalava et al., 1993) as well as bisindolylmaleimide sensitive differentiation induced by TPA (Heikkila et al., 1993). We find that SH–SY5Y cells increase mRNA levels for the adenosine A2a receptor in response to TPA in a bisindolylmaleimide sensitive manner. The doses of the protein kinase C inhibitor are within the range reported for other studies (Heikkila et al., 1993; Ferry et al., 1994; Feve et al., 1995). We conclude that the increase in adenosine A2a receptor mRNA levels in SH-SY5Y cells is likely to be mediated by activation of protein kinase C. Bisindolylmaleimide modestly reduced basal levels of adenosine A2a receptor mRNA. A degree of basal protein kinase C activity may be required to sustain unstimulated adenosine A2a receptor mRNA expression.

Following treatment with phorbol esters, SH-SY5Y cells undergo morphologic and biochemical changes consistent with differentiation (Leli et al., 1992; Heikkila et al., 1993). These changes occur following phorbol ester treatment of 24–72 h. In the present study, increases in adenosine A2a receptor mRNA were first observed by 2 h and were maximal after 6 h of TPA treatment. The increased levels of adenosine A2a receptor mRNA may represent a response independent of the differentiation program elicited by TPA, or a very early event in the process of differentiation.

Alterations in levels of mRNA may result from changes in gene transcription, mRNA stability, or a combination of effects. The stability of TRH receptor mRNA is decreased when GH pituitary cells expressing the recombinant TRH receptor are treated with phorbol esters (Gershengorn et al., 1994) whereas the stability of 5-HT2A mRNA is increased by TPA in P-11 cells (Ferry et al., 1994). Phorbol esters acting via protein kinase C and the AP1 complex activate transcription of many genes, such as those for neuropeptides (Fink et al., 1991). TPA treatment of SH-SY5Y cells increased adenosine A2a receptor mRNA levels without alterating the half-life of the mRNA. This suggests that increased stability of the adenosine A2a receptor mRNA does not account for TPA-dependent increases in adenosine A2a receptor mRNA. Pretreatment with actinomycin D blocked the effect of TPA, consistent with a mechanism involving increased transcription of the adenosine A2a receptor gene. Administration of protein synthesis inhibitors also increased adenosine A2a receptor mRNA levels. Co-administration of transcription and translation inhibitors resulted in no increases in adenosine A2a receptor mRNA levels. We propose the existence of a labile repressor protein which reduces adenosine A2a receptor gene expression and a protein kinase C-sensitive mechanism that increases adenosine A2a receptor mRNA, possibly by a transcriptional mechanism. A protein which represses β2-adrenoceptor gene expression has also been suggested (Hosoda et al., 1995).

In summary, we demonstrate the expression of functional adenosine A2a receptors by the SH-SY5Y human neuroblastoma cell line. SH-SY5Y cells may provide a useful model for studying the pharmacology and signal transduction mechanisms of adenosine A2a receptors. Adenosine A2a receptor mRNA is detectable in these cells and mRNA levels regulate in response to activation of protein kinase C. This is consistent with plasticity of adenosine A2a receptor gene expression, perhaps in response to receptor mediated activation of protein kinase C by neurotransmitters, hormones or trophic factors. The adenosine A2a receptor is a potential therapeutic target in the central nervous system, particularly in diseases of the basal ganglia (Fink, 1993), in the immune system where the adenosine A2a receptor is implicated in regulating inflammatory responses (Cronstein, 1994), and in the circulatory system where adenosine regulates hemodynamics (Mullane and Williams, 1990). Modulation of adenosine receptor gene expression, in conjunction with regulating synthesis, release, reuptake or degradation of the purine transmitter, may expand the array of physiologic options for adjusting responses to adenosine.

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